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An Unusual Symbiont from the Gut of Surgeonfishes May Be the Largest Known Prokaryote

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Symbionts first reported from the gut of a Red Sea surgeonfish, *Acanthurus nigrofuscus* (family *Acanthuridae*), were subsequently described as *Epulopiscium fishelsoni*. The taxonomic position of this very large (up to 576 μm in length) microorganism has previously been designated in the literature as either uncertain or eukaryotic. We suggest that similar symbionts from Great Barrier Reef surgeonfish may be prokaryotes, which together with *E. fishelsoni* from the Red Sea may represent the largest known forms of this cell type. Features identifying the symbionts as prokaryotes include the presence of bacterial-type flagella and a bacterial nucleoid and the absence of a nucleus or any other membrane-bound organelle.

An investigation of the gut contents of a Red Sea surgeonfish, *Acanthurus nigrofuscus* (family *Acanthuridae*), revealed the presence of a highly unusual microorganism (9). This endosymbiont was subsequently described as *Epulopiscium fishelsoni* and tentatively assigned to the eukaryote kingdom *Protoctista* (15). Examination of the gut contents of herbivorous and detritivorous surgeonfish from the Great Barrier Reef, Australia, revealed similar microorganisms which exhibited a range of sizes, shapes, and modes of cell division (5). Our initial ultrastructural examination of this Great Barrier Reef material indicated that these organisms may in fact be prokaryotes, not eukaryotes as previously thought (15, 16). To resolve this uncertainty we undertook a more detailed examination of the largest of the Great Barrier Reef endosymbionts, which will subsequently be referred to as epulos. Our electron microscope observations, presented below, suggest that the epulos are prokaryotes. Since we and others (15) have so far been unable to maintain epulos in pure culture, biochemical techniques are rendered problematical.

MATERIALS AND METHODS

Large epulo specimens (70 to 576 μm in length) were obtained from the host surgeonfish species *Acanthurus lineatus*, *A. nigrofuscus*, and *A. triostegus*. Smaller epulos (30 to 50 μm in length) were obtained from *Zebrasoma veliferum*, also a member of the family Acanthuridae. Surgeonfish were collected by spear at Lizard Island, Great Barrier Reef, Australia, in 1987 and 1988. All material was processed within 2 h of capture, during which time epulos retained motility. For light microscope preparations, samples of gut contents were removed and fixed in 4% formaldehyde in seawater. For thin-section preparations, samples of gut contents were removed and fixed in 2.5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) in 20% seawater for 30 min at 25°C. This material was then postfixed in 1% osmium tetroxide in the same buffer as above, also for 30 min at 25°C. The material was embedded in Spurr's resin and then sectioned and stained with uranyl acetate and lead citrate. The sizes of the epulos examined with the electron microscope in this study varied between 200 and 400 μm, making them visible through a dissecting microscope when embedded in the block. It was thus possible to select a particular specimen prior to sectioning.

For freeze fracturing the material was fixed in 2.5% glutaraldehyde in 0.2 M cacodylate-buffered seawater, glycerinated, and freeze fractured by the method of Bullivant and Ames (3). Epulos were negatively stained with 2% uranyl acetate at pH 7.2. The light photomicrograph was of an unstained sample with Nomarski interference contrast.

RESULTS

It is not possible to present an electron micrograph of a longitudinal section at a magnification showing both the complete organism (which on the Great Barrier Reef ranges from 70 to 576 μm for the large epulos) and sufficient internal detail. For a longitudinal view a light micrograph is therefore necessary (Fig. 1). To show ultrastructural detail, we use a transverse section (Fig. 2). The organism has a mat of flagella on its surface. Light microscope video images of epulos swimming show a pulsatile layer of liquid movement in a narrow layer corresponding to this mat of flagella. Indeed, moving epulos appear to have waves passing over their surfaces, and these waves change as swimming direction is reversed.

Beneath the outer surface is a peripheral layer of convoluted membranes, followed by a dense region with large, lightly stained inclusions. In the central region of the cell there is often a membrane-bound, dark-staining area (Fig. 2). This region has been previously referred to as a daughter cell (5, 9, 15) and is involved in the reproductive process. Upon completion of development, the daughter cells emerge through a centrally located, oblong split in the envelope of the mother cell (15).

Epulos possess bacterial-type flagella (22) rather than eukaryote-type cilia. The epulo flagella have a diameter of 14 to 18 nm in thin section (Fig. 3) and in freeze fracture profile (Fig. 4), compared with 200 to 250 nm for eukaryote cilia (8). It has not been possible to negatively stain the flagella of very large epulo specimens. When dried down in negative

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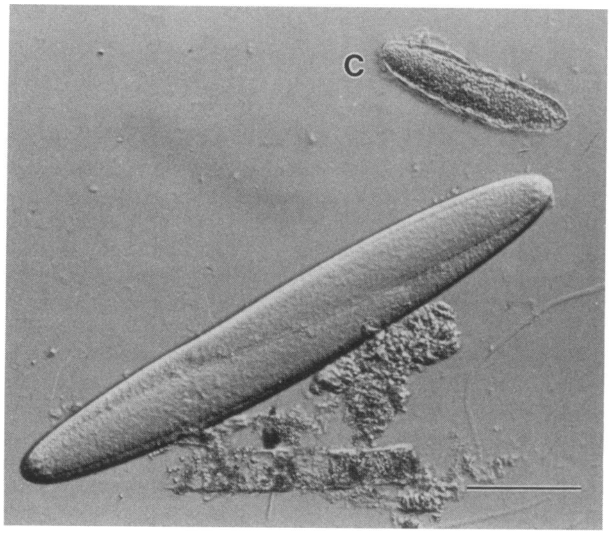

FIG. 1. Light micrograph of a large epulo and a ciliated eukaryote (C) from *A. nigrofuscus*. The ciliated cell is a protozoan of the subphylum Opalinata (10). Twin daughter cells are clearly visible within the epulo cell, which is 533 μm in length. Bar = 100 μm.

FIG. 3. Electron micrograph of thin section of an epulo from *A. triostegus* showing a peripheral convoluted membrane layer (top) within the cell and bacterial-type flagella projecting into the surrounding space (bottom). Bar = 0.5 μm.

stain, the flagella originating on the top surface of the organism adhered to that surface and did not reach down to the support film. Those flagella on the undersurface of the organism were obscured by the overhang. The flagella of similar but smaller epulos (Fig. 5) do show the characteristic helical subunit arrangement typical of bacterial flagella (13). No eukaryote-type cilia with a 9+2 microtubule structure have been observed by us or previous workers (9, 15).

Epulos possess bacterial-type nucleoids rather than eukaryote-type nuclei. The nucleoid DNA is found in circumscribed regions scattered throughout the mother cell cytoplasm, while in the daughter cells it is often found as a concentric peripheral region. Its peripheral distribution is atypical for bacteria (11, 20) but may be related to the maturation of the daughter cell. It has a coagulated appearance (Fig. 6) typical of that seen in the bacterial nucleoid after standard fixation for electron microscopy (11, 20). The nucleoids do not have a surrounding membrane. The central region of the daughter cell contains many ribosomes, typical of bacterial cytoplasm in this regard, although its appearance is more heterogeneous than is usually seen. The limiting membranes of the daughter-cell do not show any structures resembling nuclear pores (8), either by freeze fracture (Fig. 7) or in thin section.

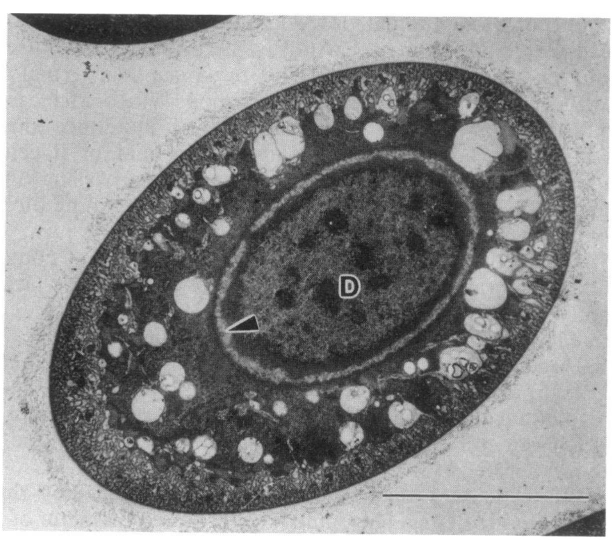

FIG. 2. Electron micrograph of thin transverse section of an epulo from *A. triostegus*. See text for a description. In the daughter cell (D) the concentric peripheral nucleoid region is indicated by an arrowhead. Bar = 10 μm.

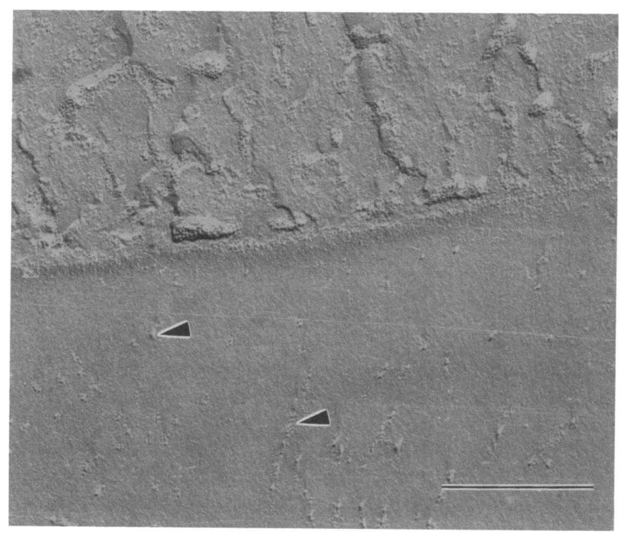

FIG. 4. Electron micrograph of freeze fracture replica of a region similar to that shown in Fig. 3, but from an epulo from *A. lineatus*, again showing a convoluted membrane region (top) and flagella (bottom). Both cross (left arrowhead) and longitudinal (right arrowhead) fractures of flagella can be seen. Bar = 0.5 μm.

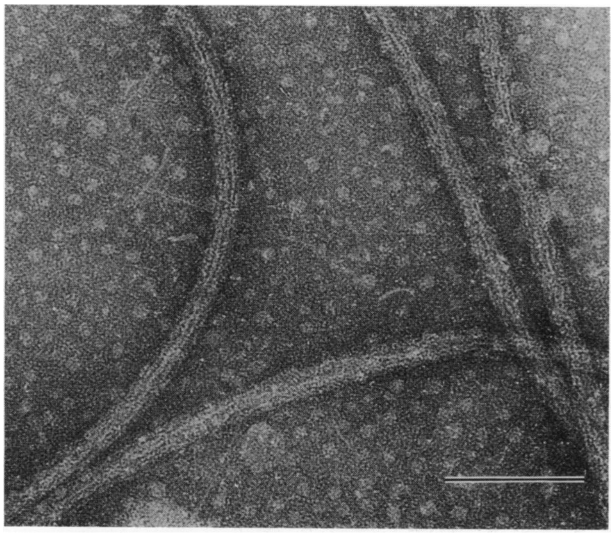

FIG. 5. Electron micrograph of negative-stain preparation of a small epulo from *Z. veliferum*, showing bacterial-type flagella. Bar = 100 nm.

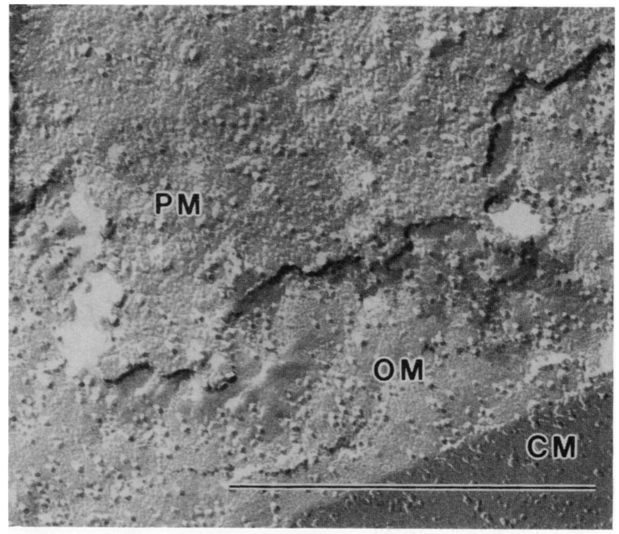

FIG. 7. Electron micrograph of freeze fracture of an epulo from *A. lineatus* showing mother cell cytoplasm (CM) and the fracture faces of the plasma membrane (PM) and outer membrane (OM) of the daughter cell. Bar = 0.5 μm.

DISCUSSION

Definitive statements on the maximum size of prokaryote cells are scarce in the literature. Despite this, a maximum size of prokaryotes is generally assumed in discussions of the evolution of eukaryote cells (4, 19). Among extant prokaryotes, *Spirochaeta plicatis* reaches maximum cell lengths of 250 μm, but such cells are only 0.75 μm in diameter (2). *Lyngbya majuscula* cells are extremely flattened disks and may be as large as 80 by 8 μm (7). Individual cells of *Beggiatoa gigantea*, a disk-shaped eubacterium, may attain a diameter of 55 μm and a width of 13 μm (23). A recent report described an unusually large *Beggiatoa* sp. from a hydrothermal deep-sea vent site (12). Filaments of these organisms attained 116 to 122 μm in diameter. However, these cells contained only a small amount of cytoplasm distributed around the outer cell wall, with the inner space of the cells filled by a large liquid vacuole (12). Other large prokaryotes include spherical cells of the photosynthetic *Prochloron* species, which are up to 30 μm in diameter (6); *Achromatium oxaliferum* cells, which are up to 35 by 100 μm (23); *Macromonas mobilis* cells, which are 6 to 14 μm wide and 10 to 30 μm in length (23); and *Thiovulum majus* cells, which have a diameter of 5 to 25 μm (23). Even a moderately sized epulo (200 by 40 μm) has a cell volume almost 10 times that of the largest of the other prokaryotes listed above.

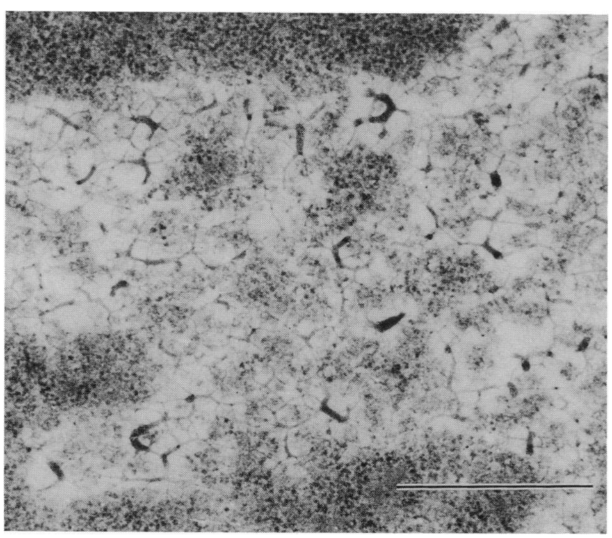

FIG. 6. Electron micrograph of thin section of nucleoid region in daughter cell of an epulo from *A. triostegus*. The coagulated appearance characteristic of bacterial DNA after standard fixation for electron microscopy is seen. Bar = 0.5 μm.

Two related factors are thought to be involved in constraining the size of the prokaryote cell: (i) the absence of intracellular transport mechanisms other than diffusion (4, 23) and (ii) the organization of DNA replication and its control (4, 7). Our ultrastructural observations show that the large symbionts possess features which may enable them to circumvent these limitations. The peripheral layer of highly convoluted membranes may represent infolding of the plasma membrane. Such infolding would vastly increase the surface area of the cell and enhance transport across the membrane into the interior. In addition, the peripheral convoluted layer may form the large compartment necessary to accommodate the proton pool (14) powering the numerous flagella needed to propel such a large organism. Finally, this convoluted membrane layer may also be involved in the coordination of flagellar action which results in the waves seen in the adjacent liquid.

The finding that epulos may be giant prokaryotes raises the question of their phylogenetic status. The prokaryote-eukaryote dichotomy represents an organizational, rather than phylogenetic, distinction (25). The phylogenetic status of epulos may be resolved by rRNA sequence analysis, which has been widely used to assess the evolutionary relationships of microorganisms (18, 21, 24). A definitive rRNA analysis of Great Barrier Reef epulos is now under way but is complicated by the lack of pure culture (17).

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ADDENDUM

Recent small-subunit rRNA sequence comparisons, to be described separately, place the epulos among the prokaryotes, as eubacteria (sensu Woese) (1).

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